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## Effects of insulin-like growth factor-I on the maturation of metabolism in neonatal rat cardiomyocytes

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**Abstract** Myocardial metabolism shifts during the perinatal period from predominant utilization of glucose towards oxidation of fatty acids. Expression of enzymes of the fatty acid oxidation (FAO) pathway is under the control of the nuclear receptor/transcription factor peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ). Insulin-like Growth Factor-I (IGF-I) plays an important role in the post-natal growth and differentiation of the heart. We determined the influence of IGF-I on the maturation of myocardial metabolism. In neonatal rat cardiac myocytes, expression of the FAO enzymes MCAD and M-CPT I was induced by treatment with the specific PPAR $\alpha$  agonist WY-14643. Concomitant treatment with IGF-I enhanced the expression of both FAO enzymes. By comparison, treatment with FGF-2, which is required for myocyte differentiation of cardiac precursors, did not increase WY-14643-induced expression of FAO enzymes. Despite stimulation of FAO enzyme expression, IGF-I did not further enhance WY-14643-stimulated palmitate oxidation. In contrast, IGF-I relieved WY-14643-mediated inhibition of glucose uptake and promoted storage of fatty acids into cellular neutral lipids. In conclusion, IGF-I promotes a more mature pattern of FAO gene expression but, because of insulin-like metabolic effects, does not concomitantly enhance oxidation of fatty acids.

**Keywords** Insulin-like growth factor-I · Fatty acid oxidation · Peroxisome proliferator-activated receptor  $\alpha$  · Glucose metabolism · Cardiac myocytes

### Introduction

Cardiac myocytes use a variety of substrate for energy production, including free fatty acids, glucose, lactate, and ketone bodies. Substrate selection is developmentally regulated. During the perinatal period, substrate metabolism shifts from predominant glycolytic and oxidative utilization of carbohydrate to predominant fatty acid oxidation [4, 20]. This shift is associated with a change in the expression of glucose transporters isoforms [25, 28, 32] and up-regulation of proteins involved in fatty acid oxidation (FAO) including fatty acyl-CoA dehydrogenases [19, 24] and carnitine palmitoyltransferases [5]. Expression of FAO enzymes, such as the medium chain acyl-CoA dehydrogenase (MCAD) or the muscle-type carnitine palmitoyltransferase I (M-CPT I), is under the transcriptional control of the nuclear receptor peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) [6, 15]. Transcriptional activity of PPAR $\alpha$  is induced upon binding of its ligand(s), most likely fatty acid derivatives. Thus, increased expression of FAO genes in the postnatal period is interpreted as a response to consumption of maternal milk rich in fatty acids. In addition to ligand binding, PPAR $\alpha$  activity is differentially regulated by phosphorylation by mitogen-activated protein kinases (MAPK). Indeed, ERK1/2-mediated phosphorylation of PPAR $\alpha$  reduces its transcriptional activity, whereas phosphorylation by p38 MAPK increases it [2, 3]. Therefore growth factors that stimulate MAPK signaling may participate in the regulation of PPAR $\alpha$  transcriptional activity and of FAO genes expression.

Insulin-like Growth Factor I (IGF-I) is believed to play a role in the postnatal growth and maturation of the heart. IGF-I is locally produced in the heart in response to growth hormone [17], the receptor of which is rapidly over-expressed in the heart after birth [22]. In addition to the promotion of hypertrophic growth of cardiac myocytes in culture [18], IGF-I elicits a more differentiated phenotype in adult rat cardiac myocytes [10]. IGF-I has been shown to stimulate expression of CPT I [16] and of the insulin-sensitive glucose transporter GLUT4 [23] in cardiac myocytes, indicating that IGF-I may favor expression of

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a more mature pattern of metabolic gene expression. Furthermore, IGF-I activates p38 MAPK in cardiac myocytes [23], which makes it a candidate regulator of PPAR $\alpha$  activity.

In the present study, we investigated whether IGF-I could enhance the expression of FAO genes induced by PPAR $\alpha$  agonists in neonatal rat cardiac myocytes (NRCs). In this model, baseline expression of FAO enzymes is rather low and readily inducible by exposure to fatty acids or PPAR $\alpha$ -specific agonists [31], thus recapitulating the in vivo postnatal transition. The effects of IGF-I were compared with those of FGF-2, a growth factor required for myocyte differentiation of cardiac precursors [27], which is also able to activate diverse MAP kinase pathways in cardiac myocytes [23].

## Materials and methods

### Animals

We obtained newborn Sprague–Dawley rats (1–3 days) from the Geneva University School of Medicine animal facility. The ethical committee of the Geneva University School of Medicine and the Geneva State Veterinary Office approved the study protocol, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### Neonatal rat cardiomyocytes culture

Neonatal rat cardiomyocytes (NRCs) were isolated by collagenase digestion and were plated (day 1) at a density of  $2.5 \times 10^5$  cells per 3.5-cm dish or  $5 \times 10^5$  cells per 6-cm dish in plating medium made of Dulbecco's modified Eagle's medium (DMEM): M199 (4:1) supplemented with 10% horse serum (HS) and 5% fetal calf serum (FCS) [30]. For transfection, NRCs were placed the next day (day 2) in DMEM: M199 (4:1) supplemented with 4% horse serum. Stimulation with agonists took place on day 3 in DMEM: M199 (4:6) containing 0.5% FCS and 10 mM creatine. All media-contained penicillin (100 U/ml), streptomycin

(0.1 mg/ml), and 100  $\mu$ M cytosine- $\beta$ -D-arabinofuranoside to prevent fibroblast growth.

Recombinant human Fibroblast Growth Factor-2 (FGF-2, Boehringer-Mannheim) and recombinant human Insulin-like Growth Factor-I (IGF-I, Bachem, Basel, Switzerland) were added from stock solutions in 10 mM acetic acid. WY-14643 (BIOMOL, Plymouth Meeting, PA) was added from a 100 mM stock solution in dimethyl sulfoxide (DMSO). The final concentration of DMSO in culture medium was 0.1%. In some experiments, DMEM: M199 was supplemented with 0.25 mM palmitic and 0.25 mM oleic acids, each complexed to 0.2 mM BSA, as described elsewhere [9]. All treatments were administered for 48 h before determination of endpoints.

### Plasmids and NRCs transfection

To indirectly determine PPAR $\alpha$  transcriptional activity, NRCs were transfected with the plasmid DR1-Luc encoding *Photinus* luciferase under the control of a synthetic promoter containing five DR1 repeats, DR1 being the core consensus sequence within PPAR response elements [1]. Christiana Juge-Aubry kindly provided the DR1-Luc plasmid. DR1-Luc was cotransfected with a plasmid-encoding *Renilla* luciferase under the control of the SV40 promoter (pRL-SV40, Promega). Transient transfection of NRCs was performed using the calcium phosphate precipitation method [30]. Transfection mixes contained 1.5  $\mu$ g of DR1-Luc and 1.5  $\mu$ g of pRL-SV40. *Photinus* and *Renilla* luciferase activity was measured 2 days after stimulation with agonists with the Dual Luciferase Reporter kit (Promega).

### RT-PCR analysis

NRCs in one 6-cm dish were extracted in 1 ml TRIZOL (Life Technologies), and total RNA was purified according to the manufacturer's protocol. Total RNA (100 ng) was treated with DNase I (Invitrogen) and reverse transcribed with the SuperScript II reverse transcriptase (Invitrogen). After degradation of the RNA with RNase H (Invitrogen), the cDNA was used for real-time polymerase chain

**Table 1** RT-PCR primers and probes

#### MCAD

Forward: 5'-TGGCATATGGGTGTACAGGG-3'

Reverse: 5'-CCAAATACTTCTTCTTCTGTTGATCA-3'

Probe: 5'-FAM-AGGCATTTGCCCAAAGAATTTGCTTC-TAMRA-3'

#### M-CPT I

Forward: 5'-ATCATGTATCGCCGCAAAC-3'

Reverse: 5'-ACCCATGTGCTCCTACCAGAT-3'

Probe: 5'-FAM- TCAAGCCGGTAATGGCACTGGG-TAMRA-3'

#### Cyclophilin

Forward: 5'-CTGATGGCGAGCCCTTG-3'

Reverse: 5'-TCTGCTGTCTTTGGAACTTTGTC-3'

Probe: 5'-FAM-CGCGTCTGCTTCGAGCTGTTTGCA-TAMRA-3'

reactions set up with the iQ Supermix kit (Bio-Rad) and run on an iCycler thermal cycler (Bio-Rad). Cycling conditions were as follows: 2.5 min at 95°C for polymerase activation followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Detection of PCR products, based on the TaqMan technology, was performed by the iCycler iQ detection and software system (Bio-Rad). Primers and probes sequences are given in Table 1. The expression of MCAD and M-CPT I was normalized for that of the housekeeping gene cyclophilin.

### Metabolic studies

Uptake of 2-deoxy-D-glucose (2-DG) in cardiomyocytes was measured, as previously described [11]. The cardiomyocytes were first incubated for 30 min in glucose-free E medium (128 mM NaCl, 6 mM KCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.4 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 2 mM sodium pyruvate, 2% fatty acid-free BSA and 10 mM HEPES pH 7.4) then incubated for 1 h in E medium containing 2-[6-<sup>3</sup>H]-deoxy-D-glucose (1 µCi), at a final 2-DG concentration of 5.75 nM. 2-deoxy-D-glucose uptake was stopped by the addition of phloretin to a final concentration of 0.4 mM. The dishes were then quickly washed thrice with ice-cold phosphate buffer saline before the cells were dissolved in 0.1 N NaOH and 2-deoxy-D-glucose trapped in the cells measured by scintillation counting.

Oxidation of palmitate was estimated from the release of <sup>14</sup>CO<sub>2</sub> from [1-<sup>14</sup>C]-palmitate. Cells were incubated in sealed flasks in 1 ml E medium containing 0.05 mM oleate and 0.05 mM [1-<sup>14</sup>C]-palmitate (1 µCi/ml) for 1 h. Reaction was then stopped by the injection of 500 µl 2N HClO<sub>4</sub> through the flask's silicon cap. The released <sup>14</sup>CO<sub>2</sub> was collected overnight on a filter paper soaked with an organic base (NCS-II, Amersham) suspended within the flask and was measured by scintillation counting.

Accumulation of neutral lipids was determined by Oil Red O staining. Briefly, NRCs were fixed with 4% paraformaldehyde in ice-cold PBS for 1 h, washed with water and stained for 2 h with 0.2% Oil Red O in 60% isopropanol. After three water washes, cells were examined by phase-contrast microscopy and pictures were acquired with a digital camera.

### Statistics

Statistical analyses were performed with the Prism4 software (GraphPad Software). Data are presented as mean±SEM. Multiple groups were compared by ANOVA, followed by Bonferroni's post-hoc test. Differences were considered significant when *p* was less than 0.05.

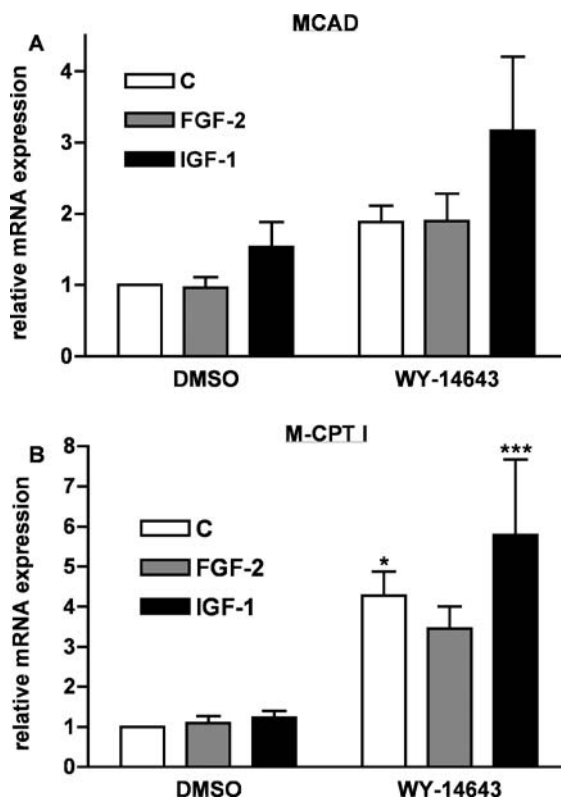
## Results

### Effects of WY-14643 and growth factors on expression of FAO enzymes

Figure 1 depicts expression of the FAO enzymes medium-chain acyl-CoA dehydrogenase (MCAD) and muscle-type carnitine palmitoyltransferase I (M-CPT I) in NRCs treated with the PPARα-specific agonist WY-14643 and growth factors. Similar to previous observations [31], WY-14643 increased expression of both FAO enzymes. FGF-2 and IGF-I had no effect on the expression of MCAD or M-CPT I in the absence of PPARα stimulation. However, IGF-I further increased the expression of FAO enzymes induced by WY-14643, whereas FGF-2 had no apparent effect.

### Effects of growth factors on PPARα-controlled transcription

Expression of M-CPT I and MCAD is under the control of PPARα transcriptional activity in cardiac myocytes. We indirectly determined PPARα transcriptional activity by



**Fig. 1** Effect of WY-14643 and growth factors on expression of FAO enzymes. Neonatal rat cardiomyocytes (NRCs) were stimulated for 2 days with either 100 µM WY-14643 or the vehicle (DMSO) in the presence of 25 ng/ml FGF-2 (grey bars), 100 ng/ml IGF-I (black bars) or the vehicle (c; acetic acid, final concentration 10 µM, white bars). Total RNA was extracted and expression of MCAD mRNA (a) and M-CPT I mRNA (b) determined by QRT-PCR. Results are the mean±SEM of 11–13 separate experiments. In each experiment, the lowest value was arbitrarily set to 1. \**p*<0.05; \*\**p*<0.01; \*\*\**p*<0.001 vs. DMSO

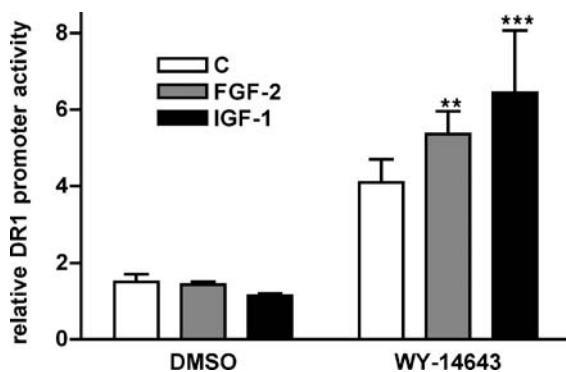
transfecting NRCs with a plasmid-encoding luciferase under the control of a synthetic promoter containing five DR1 repeats, DR1 being the core consensus sequence within PPAR response elements [1]. FGF-2 and IGF-I, in the absence of PPAR $\alpha$  stimulation, had no effect on luciferase expression (Fig. 2). WY-14643 markedly stimulated luciferase expression, indicating increased PPAR $\alpha$  transcriptional activity. Similar to the observations on M-CPT I and MCAD expression, IGF-I further increased luciferase expression. The effect of FGF-2 was, however, slightly different from that on FAO gene expression, as FGF-2 slightly enhanced transcription from the DR1 promoter activated by WY-14643.

#### Effects of WY-14643 and growth factors on substrate metabolism

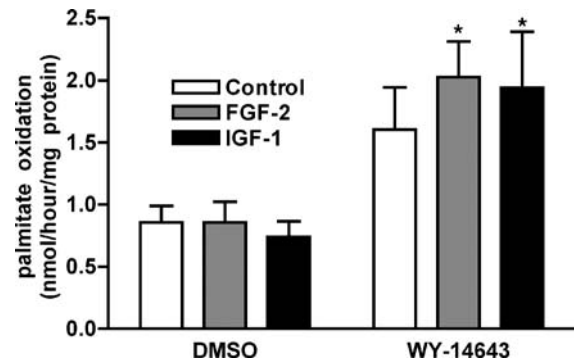
M-CPT I and MCAD catalyze rate-limiting steps in FAO. We therefore determined the influence of PPAR $\alpha$  activation in combination with growth factors on the oxidation of palmitate in NRCs. Figure 3 shows that neither FGF-2 nor IGF-I influenced the basal level of palmitate oxidation. WY-14643, as expected, markedly stimulated fatty acid oxidation; this effect was not altered by concomitant treatment with either growth factor.

Because IGF-I has insulin-like effects, we speculated that the effect of increased expression of FAO enzymes on FAO might be counterbalanced by concomitant stimulation of glucose metabolism. Indeed, WY-14643 markedly reduced 2-deoxyglucose uptake in NRCs, whereas, IGF-I partially relieved the WY-14643-induced inhibition of glucose uptake (Fig. 4).

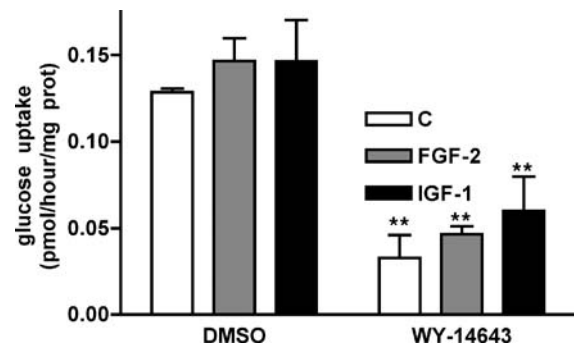
Stimulation of glucose uptake in the presence of fatty acids may promote storage of fatty acids into triacylglycerol. To determine whether IGF-I redirect fatty acids towards storage rather than oxidation in NRCs, we



**Fig. 2** Effects of growth factors on PPAR $\alpha$ -controlled transcription. NRCs were transfected with a DR1-luciferase reporter plasmid together with a pRL-SV40 plasmid for internal control and then stimulated for 2 days with either 100  $\mu$ M WY-14643 or the vehicle (DMSO) in the presence of 25 ng/ml FGF-2 (grey bars), 100 ng/ml IGF-I (black bars) or the vehicle (c, white bars). *Photinus* luciferase activity was then determined and normalized for *Renilla* luciferase activity. Results are the mean $\pm$ SEM of 13 separate experiments. In each experiment, the lowest value was arbitrarily set to 1. \*\* $p$ <0.01; \*\*\* $p$ <0.001 vs. DMSO



**Fig. 3** Effect of WY-14643 and growth factors on oxidation of palmitate. NRCs were stimulated for 2 days with either 100  $\mu$ M WY-14643 or the vehicle (DMSO) in the presence of 25 ng/ml FGF-2 (grey bars), 100 ng/ml IGF-I (black bars) or the vehicle (c, white bars). Oxidation of  $^{14}$ C-labeled palmitate was thereafter measured during 1 h. Results are the mean $\pm$ SEM of nine separate experiments. \* $p$ <0.05 vs. DMSO



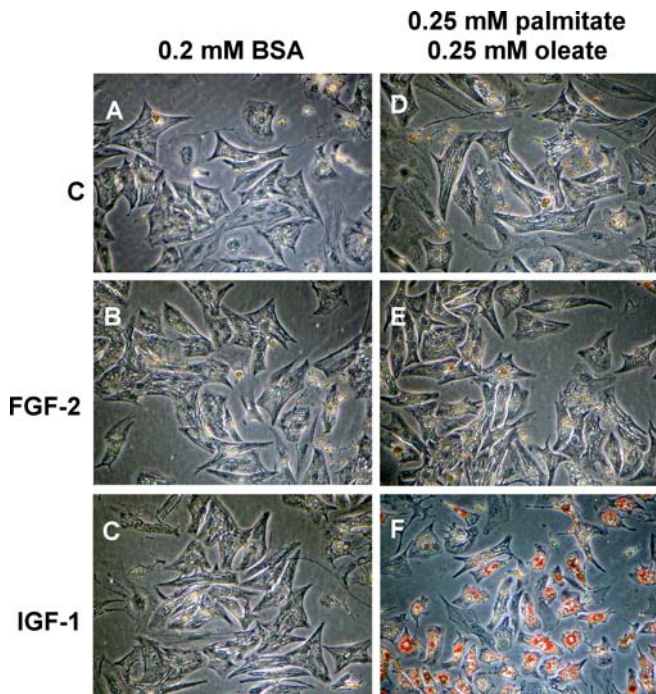
**Fig. 4** Effect of WY-14643 and growth factors on glucose uptake. NRCs were stimulated for 2 days with either 100  $\mu$ M WY-14643 or the vehicle (DMSO) in the presence of 25 ng/ml FGF-2 (grey bars), 100 ng/ml IGF-I (black bars) or the vehicle (c, white bars). Uptake of 2-deoxy-D-[ $^3$ H]-glucose was thereafter measured during 1 h. Results are the mean $\pm$ SEM of three separate experiments. \*\* $p$ <0.01 vs. DMSO

incubated NRCs for 48 h with 0.25 mM palmitate and 0.25 mM oleate together with growth factors and assessed neutral lipids by Oil Red O staining. As shown in Fig. 5f, the combination of free fatty acids (FFA) and IGF-I resulted in marked deposition of fatty acids in neural lipids storage. Oil Red O staining was much less prominent in NRCs incubated with FFA either alone or in combination with FGF-2 (Fig. 5d,e).

## Discussion

Myocardial metabolism drastically changes during the perinatal period. Indeed, while the fetal or neonatal heart mostly relies on the utilization of carbohydrates for energy production, myocardial metabolism shifts towards predominant oxidation of fatty acids within days or weeks after birth [4, 20]. This shift is associated with a marked increase in the expression of enzymes involved in fatty acid oxidation (FAO) [4, 19, 24]. In this study, we demonstrate that the postnatal surge in IGF-I may enhance the





**Fig. 5** Influence of growth factors on accumulation of neutral lipids. NRCs were incubated for 2 days in the presence of 0.2 mM bovine serum albumin (BSA) (a–c) or 0.25 mM palmitate and 0.25 mM oleate bound to BSA (d–f). Concomitantly, the cells were stimulated with FGF-2 (25 ng/ml; b and e), IGF-I (100 ng/ml, c and f) or the vehicle (a and d). Neutral lipid stores were then stained with Oil Red O and phase contrast pictures were acquired

expression of FAO enzymes; however, this effect seems to be counteracted at the substrate flux level by the insulin-like actions of IGF-I favoring utilization of glucose.

The perinatal period is a time of profound metabolic and hormonal changes in mammals. Among the hormones that control substrate metabolism, circulating insulin is very high in the preterm fetus and drops after birth, whereas, glucagon rises [14]. This results in reduced activity of myocardial acetyl-CoA carboxylase; thus, malonyl-CoA production is reduced and the inhibition of CPT I exerted by malonyl-CoA is relieved [21]. This mechanism does not, however, explain the increased postnatal expression of myocardial FAO enzymes.

Maternal milk is the only source of nutrients for newborn mammals and is akin to a high-fat diet. Thus, circulatory free fatty acid concentration, which is very low in the fetus, rapidly rises after the beginning of suckling. An important consequence thereof is activation of the peroxisome-proliferator activated receptors (PPAR) in various tissues. Cardiac myocytes express all three isoforms of PPAR, i.e., PPAR $\alpha$ , PPAR $\beta/\delta$  and, to a much lower extent, PPAR $\gamma$  [13]. Activation of the former two isoforms has been shown to regulate expression of FAO enzymes in cardiac myocytes [6, 13, 31]. Thus, the rise of circulating free fatty acids in itself may explain the increased expression of FAO enzymes in the myocardium. We speculated that endocrine mechanisms not related to nutrition might enhance this effect.

Another important hormone that rises after birth is growth hormone (GH). Tissue-specific effects of GH are often mediated through the local production of IGF-I, in an autocrine or paracrine manner. IGF-I expression itself is in the heart under the control of GH [17]. Our results suggest that local production of IGF-I, in response to GH, could participate in the increased expression of FAO enzymes in postnatal myocardium. However, we observed that IGF-I had no effects on the expression of FAO enzymes in the absence of a PPAR $\alpha$  ligand. Therefore, ligand-induced activation of PPAR $\alpha$  seems to be a prerequisite for the stimulatory effect of IGF-I on expression of FAO enzymes. This observation contrasts with a previous report by Hudson et al. [16] who observed that treatment of neonatal rat cardiac myocytes with IGF-I increased both activity and protein expression of CPT I. The reason for this discrepancy is unclear. The study by Hudson et al. relied on labeling with  $^3\text{H}$ -etomoxir for quantitative determination of CPT I protein, not discriminating between the liver (L-) or muscle (M-) isoforms, whereas we used isoform-specific primers and probes for determination of M-CPT I mRNA. It is, therefore, possible that IGF-I stimulated the expression of L-CPT I, as this isoform is also expressed in cardiac myocytes [8]. Alternatively, IGF-I may stimulate the protein expression of M-CPT I through a post-translational mechanism.

IGF-I activates multiple signaling pathways in cardiac myocytes including JAK/STAT, PKC, ERK1/2 MAP kinase, p38 MAP kinase and PI3kinase/Akt [12, 23, 29]. Of these, p38 and ERK1/2 MAP kinase have been shown to modulate the activity of ligand-bound PPAR. p38 and ERK1/2 MAP kinase phosphorylate PPAR $\alpha$  on separate residues, resulting in respectively enhanced activation [3] or inhibition [2] of the ligand-activated nuclear receptor. Thus, the net effect of IGF-I on PPAR $\alpha$  activity will depend on the relative influences of p38 MAPK- and ERK1/2-mediated phosphorylation. Our results suggest, albeit indirectly, that in the context of cardiac myocytes, the stimulatory effects of p38 MAPK activation predominates over the inhibitory effects of ERK1/2 activation. Nevertheless, DR1-driven luciferase expression was increased when the MEK1/2 inhibitor PD98059 blocked activation of ERK1/2 (data not shown), demonstrating the counterbalancing effect of ERK1/2 activation. On the other hand, we showed in a previous report [23] that inhibition of p38 MAPK with SB203580 reduced the expression of GLUT4 induced by IGF-I to a level lower than in untreated NRCs, perhaps also reflecting the counterbalancing effect of ERK1/2 activation.

Despite enhanced expression of two rate-limiting enzymes of fatty acid oxidation, M-CPT I and MCAD, IGF-I failed to further increase palmitate oxidation above the level induced by PPAR $\alpha$ -agonist treatment alone. IGF-I shares several signaling pathways with insulin [12], and, perhaps not surprisingly, we observed an insulin-like effect of IGF-I in the relative stimulation of glucose uptake. This effect was most pronounced in WY-14643-treated cardiac myocytes in which glucose uptake was markedly reduced. According to the Randle cycle hypothesis [26], increased

fatty acid oxidation reduces oxidation of glucose, explaining the reduction in glucose uptake observed in WY-14643-treated cardiac myocytes. In contrast, insulin- or IGF-I-stimulated glucose transport and glycolysis increase the supply of glycerol-3-phosphate for the synthesis of triglycerides, thereby channeling fatty acids to storage rather than oxidation. Indeed, we observed that cardiac myocytes cultured in the presence of IGF-I and physiological concentrations of fatty acids displayed more prominent neutral lipid stores than cells kept with fatty acids without IGF-I or with FGF-2.

### Study limitations

Although the effects of IGF-I in the presence of WY-14643 on enhanced expression of M-CPT I and MCAD and on glucose uptake were consistent and reproducible, they failed to achieve statistical significance. This limitation might be due to the concentration of WY-14643 used in our experiments. The dose of 100  $\mu$ M (or  $10^{-4}$  M) was selected based on a report showing stimulation of expression of enzymes of fatty acid metabolism by  $10^{-4}$  M WY-14643 in NRCs [31]. This concentration is, however, at the high end of the range used in the literature. For example, it has been observed that the inhibitory effect of WY-14643 on the endothelin-induced hypertrophy of NRCs is maximal at  $10^{-5}$  M [7]. It is, therefore, quite possible that the stimulation of PPAR $\alpha$  activity by such a dose of WY-14643 is approaching a maximum, explaining the relatively modest effects of IGF-I.

This study focused on the interaction between IGF-I and PPAR $\alpha$ -induced expression of fatty acid oxidation proteins. However, it has recently been demonstrated that PPAR $\beta/\delta$  is an important regulator of fatty acid oxidation both in neonatal [13] and adult rat cardiac myocytes (C. Pellieux, personal communication). To the best of our knowledge, no information is available on the potential regulation of liganded PPAR $\beta/\delta$  through MAP kinase-mediated phosphorylation.

In conclusion, this study indicates that IGF-I has the potential to increase expression of fatty acid oxidation proteins in cardiac myocytes through enhancement of PPAR $\alpha$  activity. At the substrates flux level, this effect is, however, counterbalanced by insulin-like stimulation of glucose metabolism.

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